

Metabolic response of cornflower (*Centaurea cyanus* L.) exposed to tribenuron-methyl: one of the active substances of sulfonylurea herbicides

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Abstract Tribenuron-methyl is the active substance of the herbicide used for weed control in crops. The aim of this study was to investigate differences in the metabolic response of seeds, seedlings and leaves of *Centaurea cyanus* L., depending on the degree of resistance to tribenuron-methyl. Changes in the values of selected biochemical and physiological parameters (germination index, chemical composition, photochemical efficiency of photosystem II and the emission spectra of blue-green fluorescence) presented herein make it possible to determine the differences between cornflower biotypes with various types of resistance to the tested herbicide. Moreover, differences in the chemical composition of dry seeds between biotypes susceptible and resistant to tribenuron-methyl were observed before using the herbicide. The degree of resistance to the herbicide—resistant or susceptible, but not the types of this resistance—mutational or metabolic, can be distinguished on the basis of the presented parameters. These findings allow for early diagnosis of the resistance of cornflower to tribenuron-methyl. Additionally, we suggest that the described parameters might be used as physiochemical markers for early estimation of weed resistance to

various types of herbicide. The presented conclusions are especially important for agricultural practice.

Keywords Tribenuron-methyl · FT-Raman spectroscopy · Photosynthetic efficiency of PSII · Fluorescence · Resistance to herbicides · Cornflower

Abbreviations

| | |
|-----------|--|
| ABS/ | Light absorption flux (for PSII antenna |
| RC | chlorophylls) per reaction center (RC) |
| Area | The area above the chlorophyll fluorescence curve between F_0 and F_m (reflecting the size of the plastoquinone pool) |
| $DI_0/$ | Dissipation energy flux |
| RC | |
| $ET_0/$ | Maximum electron transport flux (further than |
| RC | Q_A^-) |
| F_0 | Chlorophyll fluorescence intensity measured when all PSII reaction centers are assumed to be open |
| F_m | Maximal chlorophyll fluorescence intensity measured when all photosystem PSII reaction centers are closed |
| F_v | Variable chlorophyll fluorescence |
| F_v/F_0 | A value that is proportional to the activity of the water-splitting complex on the donor side of the PSII |
| F_v/F_m | A value that is related to the maximum quantum yield of PSII and specific phenomenological fluxes and quantum efficiencies |
| PI | The performance index |
| T_{FM} | Time needed to reach F_m |
| $TR_0/$ | Trapped (maximum) energy flux (leading to Q_A |
| RC | reduction) |
| Q_A | The primary quinone acceptor of PSII |

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Introduction

A common problem in the world agriculture is the acquired resistance of weeds to herbicides. In Poland, since 2010, cornflower (*Centaurea cyanus* L.) is classified to the group of weeds resistant to sulfonylurea-derived inhibitors (Heap 2015). Farmers are constantly searching for weed control solutions to improve the quality of crops. The difficulty lies in the fact that it is not known whether the weed population in a particular field is resistant to any herbicide.

Cornflower is one of the most common segetal weeds. It occurs mainly in winter cereals, rapeseed, root crops and papilionaceous plants. As civilization developed and the use of chemicals protecting crops from weeds intensified, an increasing number of weed biotypes emerged, with resistance to herbicides derived from various chemical groups (this also applies to cornflower). This process was particularly driven by compounds from the sulfonylurea herbicide family, which includes tribenuron-methyl (Heap 2015). As a consequence of this phenomenon, since 2010, cornflower *C. cyanus* has been listed among the weeds resistant to sulfonylurea inhibitors in Poland (Heap 2015).

Sulfonylurea herbicides that contain tribenuron-methyl as an active substance affect the functioning of the acetolactate synthase (ALS)/acetohydroxyacid synthase (AHAS) enzyme (Gerwick et al. 1993). In susceptible plants, these herbicides block the action of the ALS enzyme and inhibit the first stage of amino acid synthesis, thereby disturbing cell division in meristematic tissues of plants (Hess 1987; Rost and Reynolds 1985), as valine, leucine and isoleucine are involved in the regulation of cell division. The mitotic cycle is inhibited within a few hours after the application of herbicides, which in susceptible plants causes arrest in the growth of roots and aerial parts (Scheel and Casida 1985). Resistant biotypes have a higher activity of ALS/AHAS in comparison with susceptible plants, and thus the synthesis of valine, leucine and isoleucine is more intense (Marczewska et al. 2006). Although sulfonylurea derivatives do not directly inhibit the process of photosynthesis, their activity may impair the efficiency of the photosynthetic apparatus. Moreover, their presence in the environment affects the general metabolism of the plant. The effects of the herbicide on the overall metabolism of plants were shown previously in wild oat seedlings (Stokłosa et al. 2006) and rigid rye-grass (Saja et al. 2014), but using herbicides with different mechanism of action—herbicides from the group ACC-ase inhibitors. Sulfonylurea herbicides, including Granstar 75 WG, whose active substance is tribenuron-methyl, are applied to the green parts of plants, but in view of the fact that herbicide resistance is inherited, it is believed that even at the level of dry seeds there are various differences in the chemical composition of the

endosperm associated with the resistance trait. Therefore, knowledge of the physiological basis of changes caused by the action of a herbicide in plants can enable finding a specific resistance marker of the plant to the active substance of the herbicide.

In this study, we investigated the metabolic response of *C. cyanus* in relation to the degree of resistance to tribenuron-methyl, following the application of the herbicide using non-invasive methods (Raman spectroscopy and fluorescence methods). Raman spectroscopy is used in studying plant responses to stress factors, which makes it possible to track stress-induced changes in the chemical composition of tissues (Skoczowski and Troć 2013). Fluorescence methods have been used in the studies on the effects of herbicides on plants (Christensen et al. 2003) due to the quick, non-invasive and simple manner of assessing the physiological state of plants in comparison with traditional methods of resistance testing (Beckie et al. 2000).

The aim of the work was to answer the following questions:

1. Do seeds collected from cornflower biotypes resistant and susceptible to the tested sulfonylurea herbicide have different chemical composition?
2. Are there any differences in the ability to germinate as well as in the chemical composition between cornflower biotypes with varying degrees of resistance to tribenuron-methyl when it is applied to the roots (seedling experiments)?
3. Are there differences in the chemical composition and the effectiveness of photosynthesis between cornflower biotypes with varying degrees of resistance to tribenuron-methyl administered by foliar application (experiments on plants at the four-leaf stage)?
4. Are differences in the chemical composition and photosynthetic efficiency sufficiently pronounced so that they can serve as resistance markers to the active substance of the herbicide?

Materials and methods

Plant material and experimental design

The study used the seeds, seedlings and leaves of cornflower biotypes with varying degrees of resistance to sulfonylurea herbicide. The plant material originated from north-eastern and south-western Poland. Three different biotypes were studied with different degrees of resistance to tribenuron-methyl—two resistant biotypes with metabolic and mutational (Pro197) type of resistance and one susceptible biotype (control). The plant material was obtained from prof. Kazimierz Adamczewski, at the

Institute of Plant Protection National Research Institute in Poznan, where it was validated based on molecular tests. The study tested the effect of the active substance in the form of methyl 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoylsulfamoyl] benzoate, conventionally known as tribenuron-methyl. It is the active substance of Granstar 75 WG—a commercial herbicide (DuPont). On the basis of preliminary results (data not shown), two concentrations of herbicide solutions were chosen: 16 and 64-fold higher than the recommended dosage. The study was based on three approaches.

In the first experiment, cornflower seeds collected from the field, with different degrees of resistance to tribenuron-methyl, were analyzed by using Raman spectroscopy. The seeds were cut longitudinally and a single measurement was made for each half of the seed. Analyses were performed in 10 replicates on 5 seeds collected from each biotype; where the measurement was taken on one half of the seed and it was considered a single replicate.

In the second experiment, the effect of the root-applied tribenuron-methyl was studied in cornflower seedlings. The seeds were placed on a moistened filter paper in Petri dishes (3.5×4 cm), 4 dishes with 5 seeds each. Each of the plates was considered a single replication. The seeds were placed on a filter paper disc, moistened with the herbicide (64-fold higher concentration than the recommended one) and covered with an additional filter paper disc, which was also moistened with the herbicide. In the control, distilled water instead of the herbicide was used. The seeds were germinated for 7 days at 25 °C in darkness. After 7 days, germination index was determined and the seedlings were collected, lyophilized and the chemical composition of cotyledons was analyzed using FT-Raman spectroscopy. Analyses were performed on 10 cotyledons from 5 seedlings taken from each biotype.

In the third experiment, the effect of the leaf-applied tribenuron-methyl was studied in cornflower plants. Seeds of each of the biotypes (3 per biotype) were planted into 250-cm³ plastic pots. Horticultural soil mixed with sand in a 3:1 ratio was used. The pots were placed for 4 weeks in phytotron chambers under controlled conditions: temperature 25/20 °C (day/night); day length 16 h; PAR intensity 400 μmol (photons) $\text{m}^{-2} \text{s}^{-1}$; RH: 70 % (Marczewska—personal communication). The leaves were sprayed when plants reached the stage of 3–4 leaves. Spraying with a herbicide at a dosage 16-fold higher than the recommended concentration or spraying with water (control) was performed using a Kwazar Mercury 0.5-l hand sprayer. The herbicide and water were applied with the addition of Trend 90 EC adjuvant. The measurements were carried out for 10 days after spraying. At the beginning, measurements

of PSII efficiency were performed. The measurements were performed in the upper part of the fourth leaf on 10 plants of each biotype. One leaf of each plant was considered a single replicate. Then, the plant material was collected for measurements of leaf fluorescence. The measurements were carried out on the fourth leaf, in 10 plants of each biotype. The leaves were subsequently lyophilized, and their chemical composition was analyzed using FT-Raman spectroscopy. The measurements were performed on 5 leaves (two areas per leaf) from each biotype—a total of 10 replicates.

Measurements

Seed germination and germination index

The number of germinated seeds was calculated once every 24 h for 7 days. Analyses were made for 20 seeds (grown on water) in four repetitions (5 seeds per repetition). The moment of the appearance of the embryonic root was considered for the seed germinated. After 7 days the germination index (GI) was calculated using the equation described by Olofsdotter et al. (2002):

$$\text{GI} = 2(5x + 4x + 3x + 2x + x),$$

where:

x seedlings growth within each 24 h period ($5x = 24$ h, $4x = 48$ h, $3x = 72$ h, $2x = 96$ h, $x = 120$ h).

FT-Raman spectroscopy measurements

FT-Raman measurements were performed on dry seeds, cotyledons and leaves of cornflower biotypes using a Nicolet NXR 9650 FT-Raman spectrometer equipped with a Nd:YAG³⁺ laser, emitting a beam at 1064 nm wavelength, and an InGaAs detector. The measurements were made at room temperature according to the modified method developed by Saja et al. (2014). The measurements were performed at an aperture of 80 and a spectral resolution of 4 cm^{-1} . Spectra were recorded with a laser power of 0.4 W, in the range 800–1800 cm^{-1} for seeds and seedlings and 900–1700 cm^{-1} for leaves. Sixty-four scans were performed for each spectrum. The analysis of spectra was carried out using Omnic 8, OriginPro 2015 and Statistica 9.0 software packages for Windows. Table 2 presents the identification of chemical compounds assigned to particular bands in the Raman spectra. Spectra were compared using hierarchical cluster analysis according to Ward's algorithm. The analysis was performed in the range of 800–1800 cm^{-1} for seeds and seedlings and 900–1700 cm^{-1} for leaves using STATISTICA software (StatSoft, Inc. 2011).

*The fast kinetics of chlorophyll *a* fluorescence (PS II efficiency)*

Measurements of chlorophyll *a* fluorescence kinetics and energy flow through PS II were made using a Handy PEA fluorometer (Hansatech Instruments, England) as described by Skoczowski et al. (2011). Before the measurement, the leaves were acclimated to darkness for 20 min. Radiation of 3 mmol (photons) m⁻² s⁻¹ was used for the excitation of chlorophyll fluorescence. All fluorescence values were

recorded in the period from 10 µs to 1 s from the moment of illumination. Analyses were performed using PEA Plus, Microsoft Excel 2010 and Statistica 9.0 for Windows software. The calculation of kinetics fluorescence parameters and explanation of their meaning were prepared according to Strasser et al. (2000, 2004). The PSII efficiency was established on the basis of the following technical parameters: F_0 , F_m , T_{FM} , F_v , F_v/F_0 , F_v/F_m and specific phenomenological fluxes and quantum efficiencies: ABS/RC, TR₀/RC, Q_A, ET₀/RC, DI₀/RC. Moreover, the following parameters: Area and PI were also determined.

Table 1 Germination capacity of cornflower biotypes with different type of resistance to the tribenuron-methyl

| Type of resistance | Days of germination | | | | GI |
|----------------------|---------------------|----|----|-----|-----|
| | 1 | 2 | 3 | 4–7 | |
| | % Germinated seeds | | | | |
| Resistant-metabolic | 75 | 5 | 10 | 0 | 170 |
| Resistant-mutational | 55 | 15 | 0 | 0 | 134 |
| Susceptible | 15 | 60 | 0 | 0 | 126 |

Percent of germinated seeds each day and germination index after 7 days (GI) was calculated for the population of 20 seeds germinating on the water (four replications, each containing five seeds). Maximal value of GI for 20 seeds after 7 days is equal 200 (calculated by the formula of Olofsdotter et al. 2002)

Leaf fluorescence

The measurements of fluorescence emission spectra of the leaves were made, at room temperature, in the upper part of the leaf blade using a Perkin-Elmer LS50B spectrofluorometer (equipped with a front surface accessory—52123130) according to Lichtenthaler et al. (2004). The slots for the excitation and the emission beam were set to 15 nm. The blue-green fluorescence of phenols (F450 and F530) was measured in the range of 400–600 nm whereas the red and far red chlorophyll fluorescence (F690 and F735) in the range of 600–800 nm. Excitation wavelengths were set to 355 and 430 nm, respectively. FL WinLAB Version No. 3.00 and Statistica 9.0 for Windows were used

Table 2 Assignment for the most characteristic Raman bands observed for tested biotypes of dry seeds of cornflower

| Peak number | Wavenumber (cm ⁻¹) | Components | References |
|-------------|--------------------------------|--|--|
| 1 | 841 | Monosaccharides (glucose), disaccharides | Schulz and Baranska (2007), Corbett et al. (1991) |
| 2 | 872 | Monosaccharides (fructose), disaccharides, polysaccharides (amylose, amylopectin) | Schulz and Baranska (2007), Corbett et al. (1991) |
| 3 | 900 | Monosaccharides (glucose), disaccharides | Schulz and Baranska (2007), Corbett et al. (1991) |
| 4 | 933 | Polysaccharides (amylose, amylopectin) | Schulz and Baranska (2007) |
| 5 | 971 | Polyphenols | Andreev et al. (2001) |
| 6 | 1005 | Carotenoid (tetraterpenes) | Baranska et al. (2005) |
| 7 | 1081 | Polysaccharides (cellulose) | Schulz and Baranska (2007) |
| 8 | 1122 | Polysaccharides, disaccharides (celulose, sucrose) | Schulz and Baranska (2007), Barron et al. (2006) |
| 9 | 1161 | Carotenoids (tetraterpenes) | Schrader et al. (1999) |
| 10 | 1176 | Polyphenols | Andreev et al. (2001) |
| 11 | 1263 | Lipids, fatty acids | Schulz and Baranska (2007), Muik et al. (2005) |
| 12 | 1301 | Lipids, fatty acids | Schulz and Baranska (2007) |
| 13 | 1440 | Lipids/fatty acids | Schulz and Baranska (2007) |
| 14 | 1525 | Carotenoids (tetraterpenes) | Schulz and Baranska (2007), Baranski et al. (2005), Schulz et al. (2005) |
| 15a | 1572 | Chlorophylls | Schulz and Baranska (2007) |
| 15 | 1605 | Chlorophylls, flavonoids | Schulz and Baranska (2007), Schrader et al. (1999) |
| 16 | 1654 | Amide I | Schulz and Baranska (2007) |
| 17 | 1745 | Polysaccharides (hemicellulose, pectin) lipids/fatty acids (C=O group of triglycerides—strong band) | Schulz and Baranska (2007) |

for operating the spectrofluorometer and analysis of the results.

Statistical approach

The results presented here are the mean values of independent biological replicates \pm SD (the number of replicates is provided in the description of individual experiments). Parametric one-way ANOVA was used to test the significance of the differences between means. A

comparison of the significance of mean value differences between the objects was performed using Tukey's test (HSD), at the level of significance $p \leq 0.05$. Statistical analysis was performed using Statistica 9.0 software for Windows.

Results and discussion

The impact of tribenuron methyl on seeds germination

The results of the estimation of seed germination of cornflower biotypes with various types of resistance to tribenuron-methyl are shown in Table 1. Most of the seeds, namely 75 and 55 %, with the biotype of metabolic and the mutational resistance type, respectively, were germinated after the first day, whereas only 15 % of the seeds of the susceptible biotype were sprouted at the same time. In the resistant biotypes (metabolic, mutational) the values of the germination index (GI) were higher than in the susceptible biotype.

Chemical composition in seeds and in tribenuron methyl treated seedlings and leaves

The differences between biotypes relating to the composition of the storage materials were clearly visible already in dry seeds. The ability of plants to survive and reproduce in response to the herbicide, and thus to develop resistance,

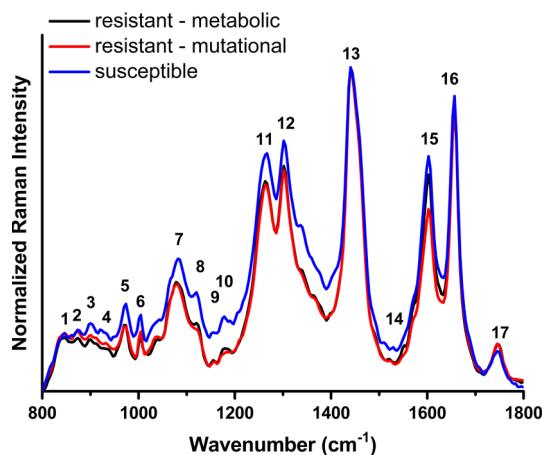
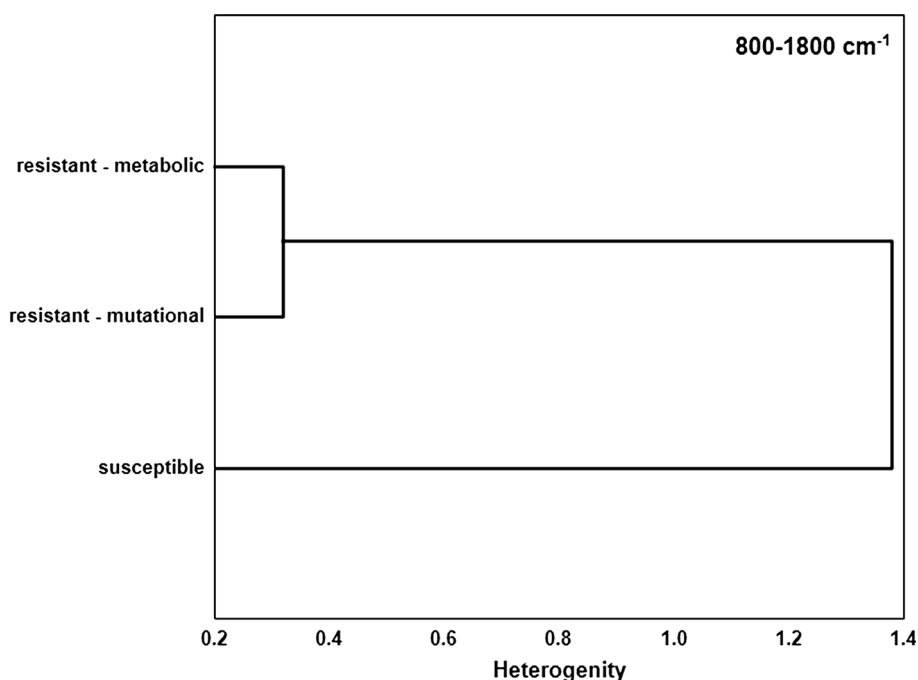


Fig. 1 Normalized FT-Raman spectra for biotypes of resistant-metabolic, resistant-mutational and susceptible to the tribenuron-methyl of dry seeds of cornflower. Meaning of the *numbers* assigned to the particular *peaks* is explained in Table 2. Spectra represents mean values from 10 replications

Fig. 2 Dendrogram showing similarities of chemical composition of dry seeds of cornflower biotypes with different types of resistance to tribenuron-methyl. Cluster analysis of the FT-Raman spectra was done by using the Ward's algorithm



might be related to their chemical composition. The chemical composition may affect the differences in the germination index between biotypes resistant and susceptible to tribenuron-methyl (Table 1). Chemical composition of seeds was determined on the basis of FT-Raman spectra. The identification of particular groups of compounds present in plant tissues was based on the specific marker bands (Table 2). Slight shifts comparing to theoretical data which were observed in some bands were due to the fact that, in plant tissue, the analyzed compounds exist together with others, which sometimes led to interference.

Analysis of the spectra obtained from dry seeds have shown the presence of marker bands, specific to several essential chemical compounds (Fig. 1). The most characteristic were those derived from mono-, di- and polysaccharides identified at 841, 872, 900, 931, 1081, 1122 and 1745 cm^{-1} . Peaks observed at 900 and 1122 cm^{-1} were, to some extent, defined by lower intensity in the susceptible biotype. The presence of lipids and fatty acids could be detected at 1263, 1301, 1440 and 1745 cm^{-1} . All the examined seeds showed a clear band at 1654 cm^{-1} arising from the presence of proteins. The slightly higher intensity of peaks for polyphenols (also flavonoids) was observed in biotypes with mutational and metabolic resistance (1176 and 1600 cm^{-1}).

In addition, hierarchical analysis of similarity was performed to compare the overall chemical composition of the studied biotypes of seeds. It was demonstrated that the chemical composition of the endosperm in biotype susceptible to tribenuron-methyl was significantly different from biotypes of metabolic and mutational resistance to this compound (Fig. 2). Different effects of tribenuron-methyl on the content of particular metabolites were observed in the cotyledons of seedlings, depending on the type of resistant biotype (Fig. 3; Table 2). Tribenuron-methyl caused an increase in the amount of almost all identified metabolites, but the greatest increase was observed for sugars, lipids and carotenoids in the biotype with mutational resistance compared to the control (Fig. 3b). Tribenuron-methyl caused a decrease, relative to the control, in the content of mono-, di- and polysaccharides (1, 2, 4, 8), polyphenols (including flavonoids—10, 15) and chlorophyll (15a) in the biotype with metabolic resistance (Fig. 3a). The increase in the content of selected compounds in response to tribenuron-methyl was most evident in the mutational resistant biotype (Fig. 3b) for carotenoids (6, 9, 14) whereas in the susceptible biotype (Fig. 3c) it was observed for sugars (1, 2, 4) carotenoids (14) and chlorophyll (15a).

The cluster analysis of cotyledons of seedlings showed significant differences in chemical composition between resistant (mutational and metabolic) and susceptible

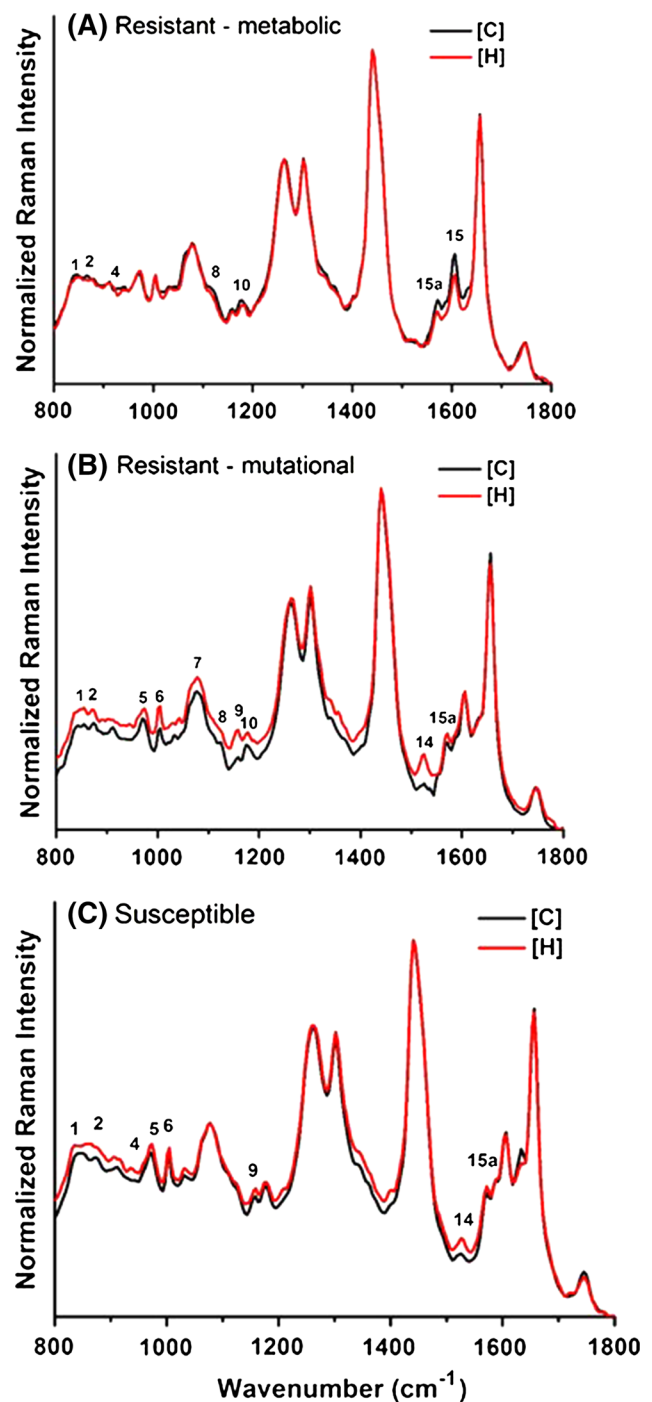
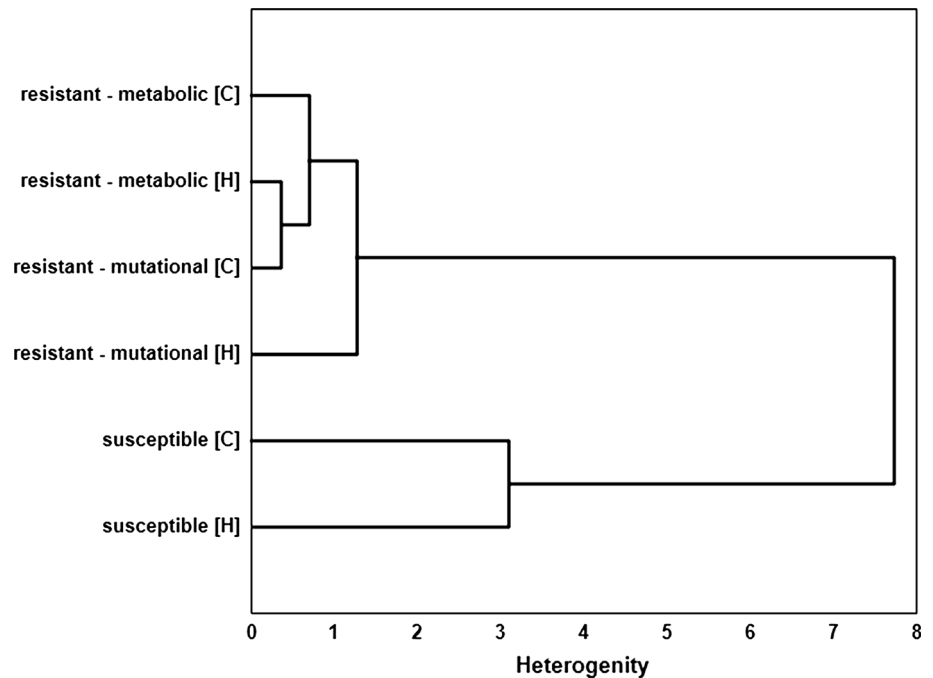


Fig. 3 Normalized FT-Raman spectra of the cotyledons of cornflower seedling biotypes with different types of resistance to tribenuron-methyl growing on distilled water (black lines) [C] and herbicide solution 64-fold higher compared to the recommended dose (red lines) [H]. Meaning of the numbers assigned to the particular peaks is explained in Table 2. Spectra represents mean values from 10 replications

biotypes, regardless of treatment ([C] or [H], Fig. 4). In addition, it was observed that each of the control objects belonging to the resistance biotypes was located on

Fig. 4 Dendrogram showing similarities of chemical composition of the cotyledons of cornflower seedling biotypes with different types of resistance to tribenuron-methyl growing on distilled water [C] and herbicide solution 64-fold higher compared to the recommended dose [H]. Cluster analysis of the FT-Raman spectra was done by using the Ward's algorithm



separate branches of the dendrogram. This indicates differences in chemical composition between the biotypes prior to the herbicide application. Furthermore, seedlings of the susceptible biotypes—both those treated with water [C] and with the herbicide [H]—were clustered in a separate group, so it can be concluded they are chemically similar. These results are consistent with the results obtained previously for rye-grass (Saja et al. 2014).

The structure of cornflowers' leaves, mainly the minimal thickness of parenchyma, caused a significant increase of the noise to signal ratio, and thus difficulties in reliable statistical analysis of all FT Raman spectra. Therefore, the chemical composition of leaves of each biotype was compared only in bands originating from flavonoids and carotenoids, because these compounds may be particularly significant in response to abiotic stress.

Given that one of the plant responses to abiotic stresses is the induction of reactive oxygen species (Bray 2002), the content of flavonoids and carotenoids in leaves was analyzed. These compounds have a significant influence on the resistance of plants (Treutter 2005) constituting an essential element of the antioxidant system of cells (Pietta 2000). The analysis of the range of FT-Raman spectra comprising bands derived from flavonoids and carotenoids is shown in (Fig. 5a–c). The presence of tribenuron-methyl results in an increase in flavonoids (1608 cm^{-1}) as well as carotenoids (carotenoid triplet— 1005 , 1161 , 1525 cm^{-1}) content in the leaves of the susceptible biotype relative to the control (Fig. 5c). Such an effect was not observed in biotypes of metabolic and mutational resistance types (Fig. 5a, b).

The cluster analysis of FT-Raman spectra obtained for leaves in the carotenoid-flavonoid range indicates that the control objects are clearly identified in the dendrogram based on the chemical composition resulting from their degree of resistance. Chemical stress caused by the herbicide differently alters the chemical composition of susceptible biotype as compared to resistant ones (metabolic and mutational) as shown by their clear separation in the dendrogram (Fig. 6). The described changes may indicate the launching of strong defensive responses in the leaves of more susceptible biotypes to counter the oxidative stress, which may occur as a side effect of the herbicide impact of the plant.

Efficiency of photosystem II of leaves treated with tribenuron methyl

This evaluation of the photosynthetic efficiency of PSII after herbicide application is of great importance in the diagnosis of resistance in weeds, where disruptions in photosynthesis occur as the second or an additional effect in the process of their destruction. An analysis of the kinetics of chlorophyll *a* fluorescence in response to the active substances of herbicides has been carried out in terms of some of the lipid biosynthesis inhibitors of the operation of acetyl-coenzyme A carboxylase (Barbagallo et al. 2003; Cowan et al. 1995), and certain inhibitors of amino acid biosynthesis—inhibitors of the operation of acetolactate synthase (Riethmuller-Haage et al. 2006). Among the analyzed parameters, the maximum photochemical efficiency of PSII— F_v/F_m was most frequently

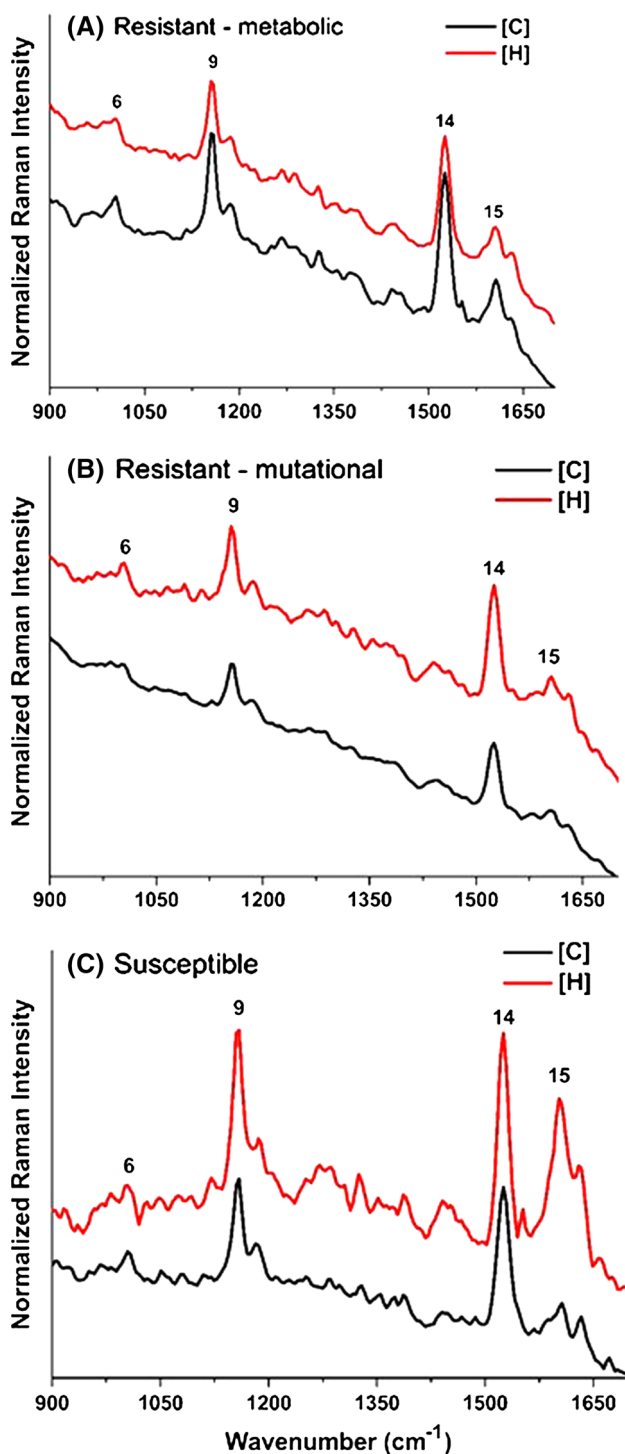


Fig. 5 Normalized FT-Raman spectra of leaves of cornflower biotypes with different types of resistance to tribenuron-methyl spraying distilled water (black lines) [C] and herbicide solution 16-fold higher compared to the recommended dose (red lines) [H]. Meaning of the numbers assigned to the particular peaks is explained in Table 2. Spectra represents mean values from 10 replications

taken into consideration. In the case of stress factors, the application of the stressor, relative to the control, demonstrated the reduction of the efficiency of energy flow in

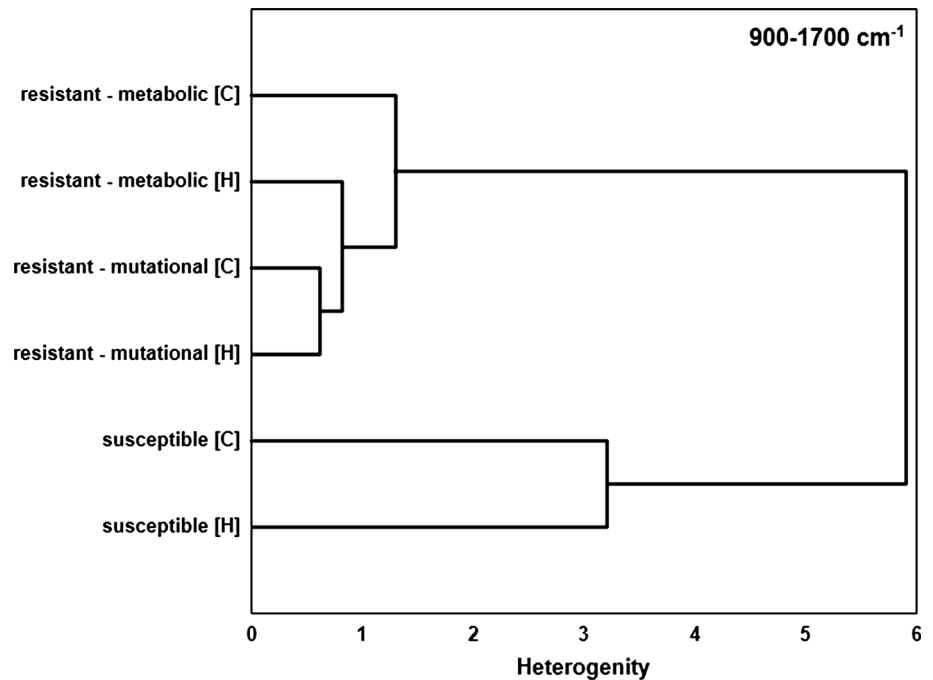
PSII (Riethmuller-Haage et al. 2006; Skoczowski et al. 2011). In the presented results, however, no significant changes in the maximum of photochemical efficiency (F_v/F_m) were observed after the application of the herbicide in contrast to the plants sprayed with water.

The results of the analysis of energy flow through PSII and the chlorophyll *a* fluorescence kinetics in leaves sprayed with the herbicide are shown in Fig. 7 as a percentage of the control. The smallest changes in the photosynthetic efficiency of PSII after treatment with tribenuron-methyl occurred in the susceptible biotype (Fig. 7c). It may indicate that susceptible biotype is, at least partially, related to the inability to increase the efficiency of the photosynthetic apparatus under stress conditions. Significant differences in the analyzed parameters after the application of the herbicide relative to the control were observed in both resistant biotypes, with the metabolic and mutational resistance type (Fig. 7a, b). In both of these biotypes, the declines in the values of PI and F_v/F_0 were accompanied by a corresponding increase in the value of the PSII antennae size indicator (ABS/RC) relative to the control. Such a chemical stress response was not observed in the susceptible biotype (Fig. 7c). However, in resistant biotypes, under conditions of impaired metabolism, the absorption of light by a single reaction center (ABS/RC) was significantly higher (by 10 % for metabolic, or even 25 % for mutational) compared to the optimal growth conditions (Fig. 7a, b). Moreover, in these biotypes a decrease in the values of the electron flow parameters (TR_0/RC and ET_0/RC) was accompanied by a decrease of the value of the DI_0/RC parameter, which indicates that less energy is dissipated as heat and may be used for electron transport (compensation for the decline in the efficiency of electron transport, Fig. 7a, b).

Fluorescence of leaves treated with tribenuron methyl

One of the non-invasive fluorescent methods is the measurement of fluorescence of leaves, which allows the diagnosis of the physiological condition of the plant to be made based on the intensity of blue-green (F450 and F530) and red fluorescence (F690 and F735) (Lichtenthaler and Babani 2004). Previous studies (Schweiger et al. 1996, Buschmann and Lichtenthaler 1998; Hideg et al. 2002) showed that the fluorescent emission spectra of leaves may be successfully used for the detection of stress in plants. One of the possibilities presenting the differences in fluorescence intensity is using the ratios of fluorescence intensity at various wavelengths F450/F520, F450/F690, F450/F740 and F690/F740. Fluorescence ratios F450/F690 and F450/F740 are relatively easy to interpret and are particularly good indicators of stress (Schweiger et al.

Fig. 6 Dendrogram showing similarities of chemical composition of the leaves of cornflower biotypes with different types of resistance to tribenuron-methyl spraying distilled water [C] and herbicide solution 16-fold higher compared to the recommended dose [H]. Cluster analysis of the FT-Raman spectra was done by using the Ward's algorithm



1996). The ratio F450/F690 was recommended by Buschmann and Lichtenthaler (1998) as a good indicator of the level of stress already noticeable in the early stages of occurrence. The value of the F690/F740 ratio is inversely proportional to the amount of chlorophyll content (Lichtenthaler and Babani 2004).

The blue-green and red fluorescence intensity spectra are shown in Fig. 8. The shape of the blue-green fluorescence spectra in the control samples of all the biotypes was similar. At a wavelength of approximately 540 nm a peak was observed, the maximum of which, in the case of the metabolic and susceptible biotypes, was approximately 0.4 relative units (Fig. 8a, c, respectively). In the mutational resistant biotype (Fig. 8b), the intensity of green fluorescence was higher and amounted to approximately 0.5 r.u. The herbicide did not have a significant effect on the changes in blue-green fluorescence intensity, but it did in resistant biotypes with metabolic and mutational resistance types only (Fig. 8a, b, respectively).

The fluorescence emission spectra of leaves showed that the herbicide significantly increased the blue-green fluorescence intensity in the susceptible biotype (Fig. 8c). The herbicide, regardless of its mechanism of action, may lead to damage of the photosynthetic apparatus of plants by lowering their resistance to intense UV radiation. The defense mechanism may consist of the accumulation of plant phenols in the leaf tissues (Bilger et al. 2001; Schmitz-Hoerner and Weissenböck 2003). In such a case, the UV radiation is converted to blue-green fluorescence (F450 and F520) which is partially re-absorbed by the assimilation pigments. It can, therefore, be assumed that the

increase in blue-green fluorescence intensity observed in the susceptible biotype under the influence of herbicide is an element of a defense strategy under which the plant protects the photosynthetic apparatus against damage by accumulating phenolic compounds in the cell walls of the epidermis.

Tribenuron-methyl decreased the intensity of red fluorescence, relative to the control, in all biotypes (Fig. 8a–c). However, while the decline was relatively small in the biotype with metabolic resistance (Fig. 8a), in the susceptible biotype the herbicide decreased the red fluorescence intensity nearly eightfold (Fig. 8c). The decrease in fluorescence intensity was accompanied by the appearance of the shoulder at 697 nm. This is particularly evident in biotypes with metabolic type of resistance (Fig. 8a). The fluorescence in the red and far red range is directly related to the content of chlorophyll in leaves; and it can be assumed that tribenuron-methyl causes disturbances in all the biotypes in the process of chlorophyll biosynthesis. This is also indicated by the emergence of the new band visible at 690 nm—F690. As the content of chlorophyll decreases, the relative intensity of red fluorescence F690 increases compared to the far red band F735. This is due to the fact that the re-absorption of red chlorophyll fluorescence decreases with its depletion. At the same time, the maximum of red fluorescence F690 shifts from longer to shorter wavelengths; the so called “blue shift” (Lichtenthaler and Babani 2004).

Tribenuron-methyl had no significant effect on the changes in fluorescence ratios relative to the control in any of the biotypes (data not shown). However, the

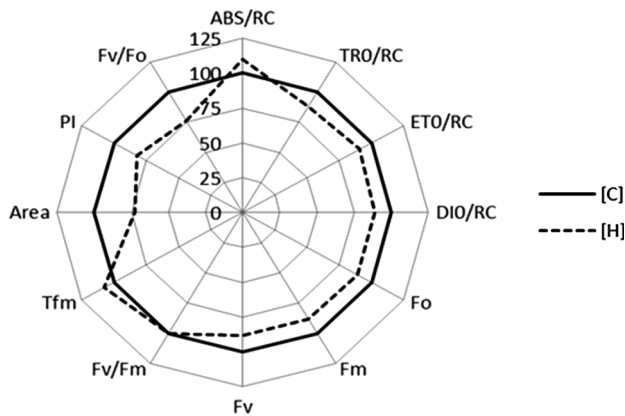
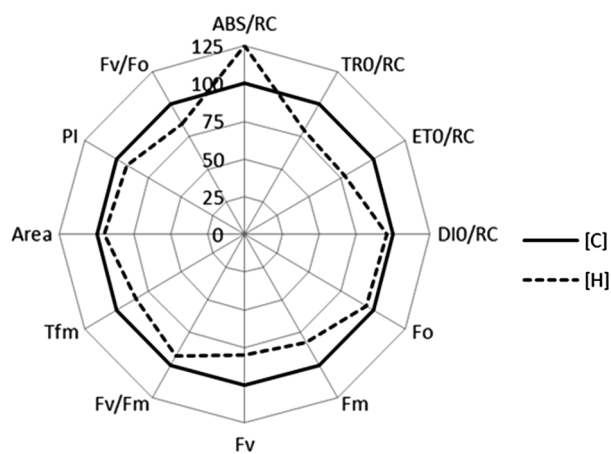
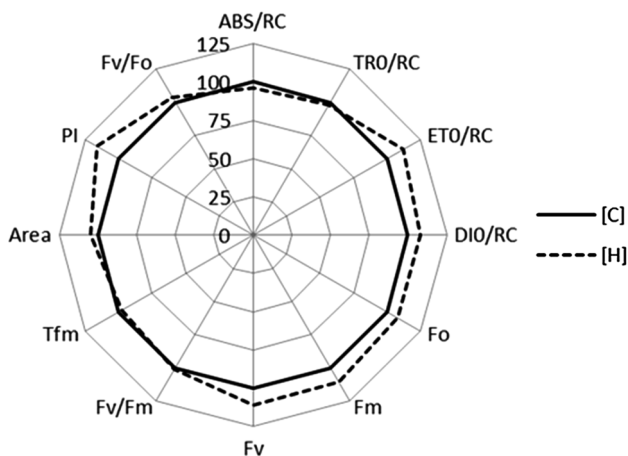
(A) Resistant - metabolic**(B) Resistant - mutational****(C) Susceptible**

Fig. 7 The parameters of chlorophyll *a* fluorescence (in % of control) in the leaves of cornflower biotypes with different types of resistance to tribenuron-methyl spraying water-control (*blue lines*) [C] and herbicide solution 16-fold higher compared to the recommended dose (*red lines*) [H]. Mean values from 10 replications

fluorescence coefficients calculated for the control plants showed that the susceptible biotype is characterized, in comparison to other biotypes, by significantly lower values of F450/F530, F450/F690 and F450/F735 ratios (Table 3). The significance of this phenomenon, at the present stage of research, is difficult to be unequivocally interpreted. However, should further studies confirm the described dependence; a comparison of the discussed fluorescence relations may be a good indicator to assess the degree of cornflower biotypes resistance to tribenuron-methyl.

Conclusions

It was demonstrated that there are physiological and biochemical differences between cornflower biotypes, connected with their different resistance to tribenuron-methyl. These differences were visible in dry seeds, seedlings and leaves both before and after application of the herbicide.

- In dry seeds, Raman spectroscopy together with the analysis of similarities allows the identification of differences in the chemical composition of the endosperm of the biotypes with varying degrees of resistance to tribenuron-methyl. It is difficult, if not impossible, to distinguish such differences by other methods. Resistant biotypes are characterized by higher germination ability as compared to the susceptible one.
- The degree of resistance of the biotype to tribenuron-methyl is reflected particularly in changes in the chemical composition of cotyledons (mainly carotenoids and flavonoids). The content of carotenoids and flavonoids in the leaves is dependent on the degree of resistance of the biotype to tribenuron-methyl. This resistance is probably also associated with the ability to acclimatize the photosynthetic apparatus to operate in stressful conditions. It has been shown that herbicides, which do not act directly on the PSII, cause disturbances in photosynthesis, and the character of the disorders depends on the biotype and its resistance. A comparison of the emission spectra of blue-green fluorescence of leaves, obtained for the plants treated with the herbicide and the control plants, allows differentiation between the biotypes of high resistance and susceptible biotypes.
- Selected biochemical and physiological parameters, obtained from the analysis of plant response to an applied herbicide, allows the inference of resistance to

Fig. 8 The fluorescence spectra of the leaves of cornflower biotypes with different types of resistance to tribenuron-methyl spraying distilled water (*blue lines*) [C] and herbicide solution 16-fold higher compared to the recommended dose (*red lines*) [H]. Spectra represents mean values from 10 replications

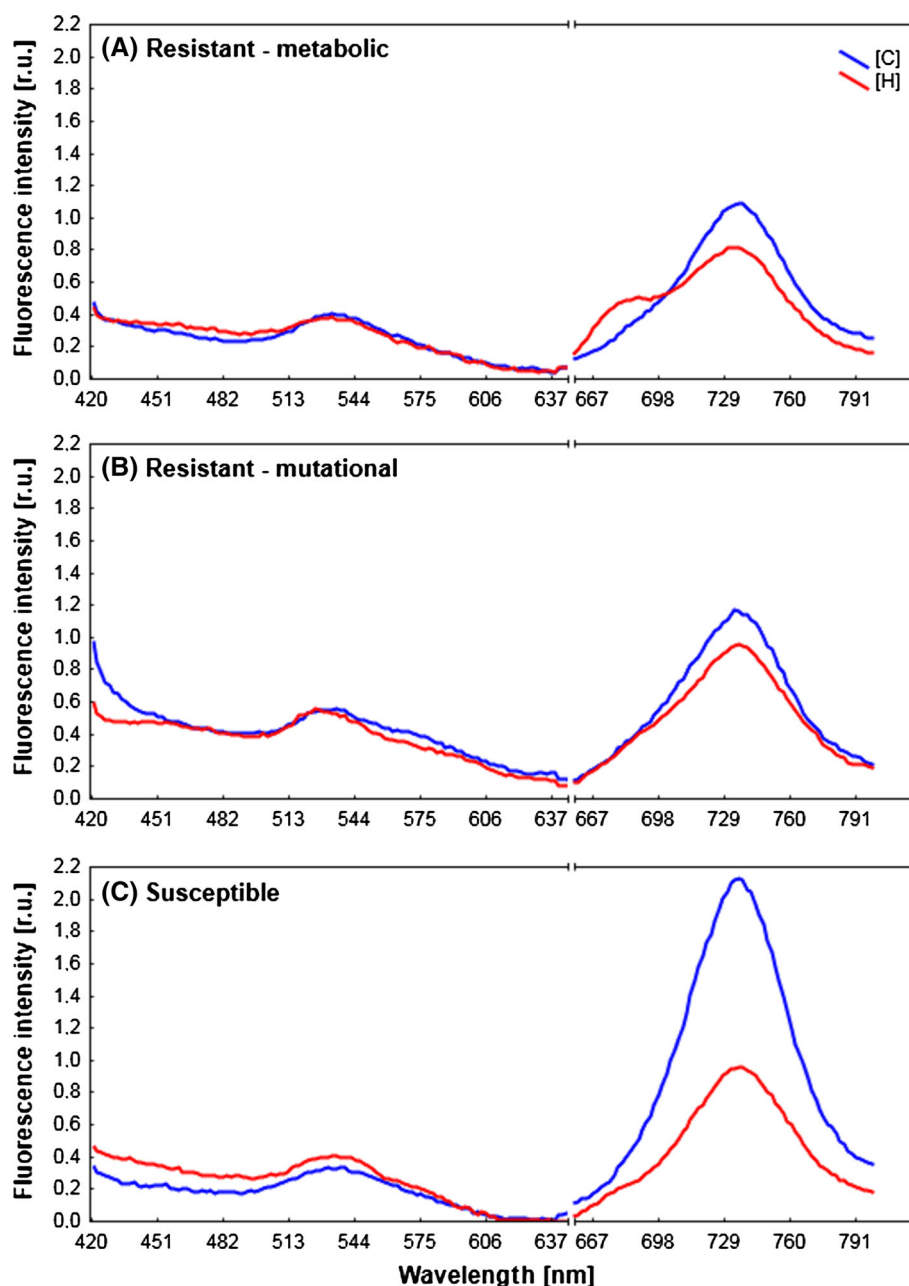


Table 3 The fluorescence ratio of leaves of cornflower biotypes with different types of resistance to tribenuron-methyl

| Type of resistance | The fluorescence ratio | | |
|----------------------|------------------------|-------------------|--------------------|
| | F450/F530 | F450/F690 | F450/F735 |
| Resistant-metabolic | 0.86 ^a | 1.34 ^a | 0.55 ^a |
| Resistant-mutational | 0.80 ^a | 1.03 ^a | 0.35 ^{ab} |
| Susceptible | 0.52 ^b | 0.17 ^b | 0.17 ^b |

The mean values of 10 replications \pm SD

^a and ^b represent statistically significant differences and ^{ab} represents lack of statistically significant differences according to Tukey's test multiple range test at $p \leq 0.05$

the herbicide. On the basis of this analysis, the degree of resistance can be distinguished (resistant or susceptible) but not the specific types of resistance (mutational or metabolic). Differences noticed between biotypes susceptible and resistant to herbicide, before using the herbicide, allow for early diagnosis of resistance. Additionally, parameters can be proposed as physiological markers for early estimation of weeds resistance to herbicide, however, this requires future studies on various species of weeds as well as various herbicides. This finding is especially important for agricultural practice.

Author contribution statement All authors contributed to write this work. D. Saja: acquisition of data, efficiency of photosystem PSII and leaf fluorescence analysis and interpretation of data, drafting of manuscript, M. Rys and I. Stawoska: FT-Raman spectroscopy analysis and interpretation of data, A. Skoczowski: study conception and design and critical revision.

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